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Full-Length Tobacco Mosaic Virus RNAs and Defective RNAs Have Different 3′ Replication Signals

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The viral replicase complex of positive-stranded RNA viruses interacts with *cis*-acting elements that are usually located at the termini of the viral RNAs. On comparison of the replication requirement of a tobacco mosaic virus (TMV)-based defective RNA (dRNA) and its helper virus, we found different requirements for replication of TMV RNAs *in cis* and *in trans*. The level of replication of full-length TMV RNA decreased substantially in the absence of pseudoknot (pk) 1 and/or 2, whereas identical deletions in dRNAs did not affect their replication. However, pk3 was required for replication of both full-length TMV RNAs and dRNAs. The requirements for homologous sequences were greater for dRNA replication than for replication of full-length TMV RNAs. Defective RNAs with heterologous 3′ nontranslated regions (NTRs) failed to be replicated or replicated minimally, whereas replication of similarly mutated full-length RNAs was much less affected. Increasing amounts of contiguous heterologous sequences in the dRNAs compensated for the impaired interactions between the replicase and 3′ NTR. The precision requirement appeared to involve the terminal 28 nucleotides, specifically the pseudoknot in the aminoacyl acceptor arm of the tRNA like structure, which was important in replication of both dRNAs and full-length TMV RNAs.

INTRODUCTION

In general, positive-stranded RNA viruses have mechanisms that prevent indiscriminate replication of cellular RNAs or heterologous viral RNAs. The viral replication complex specifically interacts with sequences or higher order structures, referred to as *cis*-acting elements, which are replication signals within the viral RNA (Chapman *et al.,* 1998; Chapman and Kao, 1999; Sivakumaran *et al.,* 1999). These replication signals are usually located near the termini of the RNA molecules. However, the different termini may have distinct roles in defining specificity. The 3′ terminal regions, which often are composed of stem-loops, pseudoknots (pks), or more complex tRNA-like structures (TLSs), tend to be conserved among the different RNAs of multipartite viruses and within virus groups. The 5′ termini have much less obvious structure and conservation (Dreher, 1999). The 3′ termini have been shown to have replication signals for initiation of complementary RNA synthesis *in vitro* and *in vivo* (for reviews see Duggal *et al.,* 1994; Lai, 1998; Dreher, 1999), although complementary RNA synthesis of several viruses has been shown to have another layer of control, a *cis*-preferential mechanism, in which the nascent replicase protein is thought to be presented to the viral RNA for initiation of replication as it completes its function as mRNA (Weiland and Dreher, 1993; Novak and Kirkegaard, 1994; Kusov *et al.,* 1996; Lai, 1998). In contrast, because progeny positive-stranded RNAs, both genomic and subgenomic RNAs, are produced in great excess over negative strands, these processes are thought to result from a pool of replicase complexes functioning *in trans*. Yet, in virus systems with multiple RNAs, satellite RNAs, or defective RNAs, the complementary RNA of at least some of these RNAs would be produced by *in trans* replicase–RNA interactions. Whether replicase interactions with replication signals are similar or different among these different types of RNAs which are produced by different strategies and perhaps different pools of replicase complexes remains obscure.

Tobamoviruses are monopartite, positive-stranded RNA viruses of approximately 6.5 kb that encode two overlapping replicase-associated proteins (126- and 183-kDa proteins in tobacco mosaic virus) produced from the genomic RNA and movement and capsid proteins produced from 3′ coterminal subgenomic mRNAs (Goelet *et al.,* 1982). The 5′ nontranslated region (NTR) of tobacco mosaic virus (TMV) consists of 68 nucleotides (nts) with little predicted structure, while the 3′ nontranslated region consists of three coaxially stacked pks (Rietveld *et al.,* 1982; van Belkum *et al.,* 1985; Leathers *et al.,* 1993)

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and a TLS that specifically charges histidine (Hall, 1979; Mans *et al.,* 1991). There is substantial variation within the pseudoknot region of tobamoviruses. The prototypical 3′ NTR represented by TMV and tomato mosaic virus (ToMV) is approximately 200 nucleotides with three pseudoknots within the 5′ region of the NTR, but several tobamoviruses have duplications of some of the pseudoknots (van Belkum *et al.,* 1985). *Odontoglossum* ringspot virus (ORSV) has a 3′ NTR of 412 nts with nine upstream pseudoknots, which includes three copies of pk2 and pk3 (Gultyaev *et al.,* 1994). Tobacco mild green mosaic virus (TMGMV) has two different forms. TMGMV U2 (U2; Siegel and Wildman, 1954) is defined as a common minor component of TMV populations that has a different phenotype, including the ability to induce necrotic local lesions in *Nicotiana sylvestris,* and TMGMV U5 (U5) is a virus that naturally infects *N. glauca* (tree tobacco) (Siegel and Wildman, 1954; Bald, 1960). The complete sequence of U2 has been determined (Solis and Garcia-Arenal, 1990), and the partial sequence of U5 is similar to that of U2, except for the 3′ NTR. U2 has a short 3′ NTR (210 nts), similar to TMV and tomato mosaic virus, with a TLS and three upstream pseudoknots, whereas U5 has a much longer 3′ NTR (357 nts) with six upstream pseudoknots (Shivprasad *et al.,* 1999; Bodaghi *et al.,* 2000).

The replication of tobamovirus genomic RNAs appears to occur primarily by a *cis*-preferential mechanism based on the inability of mutant full-length viruses to be replicated *in trans* by a competent helper virus (Lewandowski and Dawson, 1998). However, it is known that tobamovirus replicase complexes can amplify RNAs *in trans* since a satellite virus of TMGMV occurs in nature (Valverde and Dodds, 1986) and, more recently, it was found that TMV and ToMV RNAs could be replicated *in trans* if specific internal sequences were removed (Ogawa *et al.,* 1991, 1992; Raffo and Dawson, 1991; Lewandowski and Dawson, 1998).

The 3′ replication signals required for the *cis*-preferential replication of the tobamovirus genomic RNA have been identified (Takamatsu *et al.,* 1990, 1991; Watanabe *et al.,* 1996) and are contained primarily within the NTR. The minimal 3′ *cis*-acting element, which only allowed a barely detectable level of replication, consisted of the TLS plus the 3′-most pseudoknot (Takamatsu *et al.,* 1990).

Efficiently replicated TMV-based dRNAs (Lewandowski and Dawson, 1998) allow for the comparison of replication signals used by the helper virus and defective RNAs (dRNAs). Using this system, we present evidence that the requirements for replication of TMV RNAs *in cis* and *in trans* differ. (The term genomic RNAs is used to describe self-replicating RNAs which have *cis*-preferential requirements for replication and which can serve as helper RNAs for replication of dRNAs which are replicated in a *trans*-dependent manner). Optimal *in trans* replication of dRNAs required a smaller 3′ element than that of the genomic RNA, but the precision requirements for homologous sequences were greater. We also present evidence that a precise interaction between the replicase complex and the 3′ terminal aminoacyl acceptor arm of the TLS is required, plus a less precise interaction with the upstream regions which consists of the rest of the TLS and the 3′-most pseudoknot. Additional upstream sequences of the 3′ region, including the rest of the 3′ NTR, the coat protein ORF, and part of the movement protein ORF, while not required for replication, can compensate for imprecise 3′ interactions.

RESULTS

Definition of minimal and optimal 3′ terminal sequences required for replication of dRNAs

It was shown previously that tobamovirus dRNAs with large internal deletions can be replicated *in trans* by replicase complexes produced by competent helper viruses (Lewandowski and Dawson, 1998). Two of the dRNAs which accumulated to high levels were TMVΔCla (deletion of nts 1343–5664) and TMVΔHinc-Cla (deletion of nts 842–5663). These dRNAs were replicated at molar levels exceeding that of the helper virus RNA, TMV (Lewandowski and Dawson, 1998). Wild-type TMV produces large amounts of coat protein subgenomic RNA that has approximately the same size as the dRNAs, thus obscuring detection of dRNA replication. Therefore, in this study, we chose to use S3-28 as helper RNA, a TMV mutant which has the coat protein ORF deleted (Dawson *et al.,* 1988) and which has been shown to support the replication of these dRNAs to approximately the same level as wild-type TMV helper (unpublished data). Derivatives of TMVΔCla and TMVΔHinc-Cla were chosen for further examination of the 3′ *cis*-acting elements required for replication of dRNAs *in trans*.

The first objective was to define the 3′ unit of sequence that was required for optimal dRNA replication. TMVΔCla consists of the 5′ 1342 nucleotides of TMV joined to the 3′ 731 nucleotides (Fig. 1A). The 3′ segment consists of the 3′ 46 nucleotides of the movement protein ORF, the complete coat protein ORF, and 204 nucleotides that comprise the 3′ NTR. It was already known that little more than the complete 3′ NTR was sufficient to provide optimal replication of the ΔCla-based RNAs, because a dRNA with a larger internal deletion to near the 3′ NTR (Δ1344–6055; the 3′ NTR begins at nt 6193) resulted in efficient replication (Lewandowski and Dawson, 1998). Precise deletion of the coat protein ORF (Δ1343–5664; 5712–6192) leaving only the 3′ NTR (Fig. 1A) results in replication identical to that of TMVΔCla (lanes 1 and 2, Fig. 1B). A larger deletion (Δ1342–6249), removing both 3′ ORFs and extending 58 nucleotides into the 3′ NTR (Fig. 1A), resulted in slightly reduced replication of the dRNA (lane 3, Fig. 1B). However, the deletion mutant

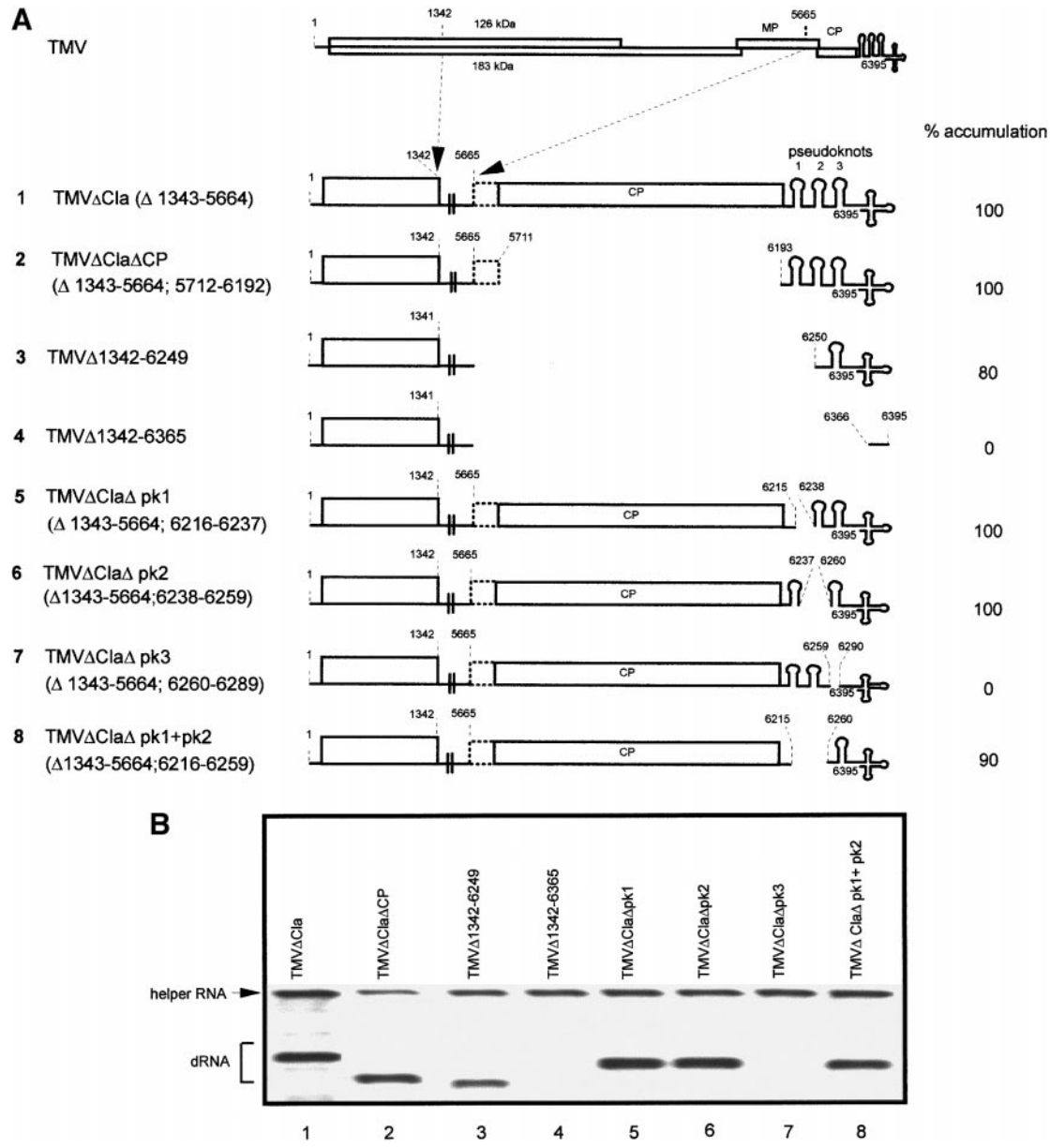

FIG. 1. (A) Schematic diagram of the genomic organization of dRNAs of TMV constructed *in vitro*. The sequences between nts 1343 and 5664 were deleted. The portion of the genome 5′ of the deletion is not drawn to the same scale as the 3′ portion. MP, movement protein ORF; CP, coat protein ORF. The deletion is shown as +. (B) Accumulation of dRNAs in tobacco suspension protoplasts. Northern hybridization blots of total RNA extracted from protoplasts 20 h postinoculation were probed with a TMV 5′ (+)-specific riboprobe. *In vitro* transcripts of pT7S3-28 were used as helper RNA. The lane numbers in B correspond to the numbers of the constructs in A. The percentages of accumulation of the different mutants compared to wild type (100%) are indicated to the right in A.

TMVΔ1342–6365, which retained only 30 nucleotides of the 3′ NTR (Fig. 1A), did not accumulate to detectable levels (lane 4, Fig. 1B). These results suggested that a subset of the 3′ NTR was sufficient to provide optimal replication of dRNAs of TMV.

The TMV 3′ NTR consists of three pseudoknots located between the coat protein ORF and the TLS, referred to as pk1, pk2, and pk3, 5′ to 3′, respectively (Fig. 2A). Since deletions into the 3′ NTR resulted in a reduction in replication levels which was determined by the extent of the deletion, precise removal of each pseudoknot from genomic RNA of TMV, singly and in combinations, was compared to identical deletions in TMVΔCla. Deletion of the pseudoknots affected replication of the full-length and dRNAs differently. Deletion of pk1 or pk2, individually, did not decrease replication of the dRNAs and deletion of both pk1 and pk2 only slightly reduced replication (by ~10%) (lanes 5, 6, and 8, Fig. 1B). However, deletion of pk1 in genomic TMV RNA reduced replication by ~10% (lane 2, Fig. 2B), and deletion of pk2 (lane 3, Fig. 2B) or pk1 + 2 (lane 5, Fig. 2B) resulted in a 70% reduction in replication. Deletion of pk3 totally abol-

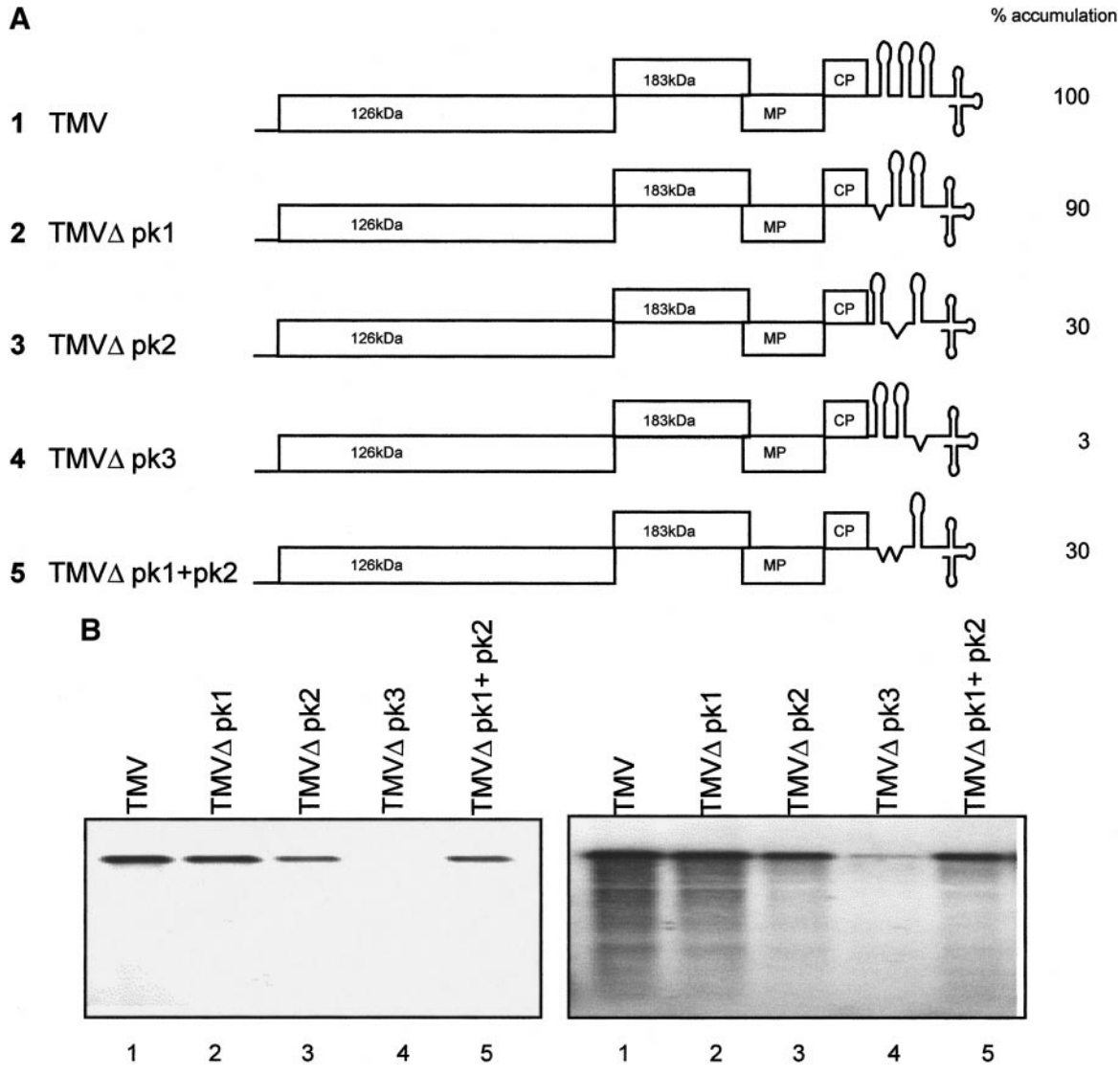

FIG. 2. (A) Schematic diagram of the genomic organization of TMV mutants with pseudoknots deleted. MP, movement protein ORF; CP, coat protein ORF; pk, pseudoknot. (B) Accumulation of genomic RNAs in tobacco suspension protoplasts. Northern hybridization blots of total RNA extracted from protoplasts 20 h postinoculation were probed as described in Fig. 1. I and II are different exposures of the same blot. The darker exposure (II) shows the low level of replication of TMVΔpk3. The lane numbers in B correspond to the numbers of the constructs in A. The percentages of accumulation of the different mutants compared to wild type (100%) are indicated to the right in A.

ished dRNA replication (lane 7, Fig. 1B), but the same deletion in full-length TMV RNA resulted in continued, barely detectable replication (lane 4, Fig. 2B). These results indicated that the TLS and pk3 were the minimal sequences required to support near-optimal levels of replication of the dRNAs, although they provided only greatly reduced replication of full-length RNAs.

Substitution of heterologous 3′ sequences into genomic RNA and TMV dRNAs

We next examined the effect of heterologous 3′ sequences on replication of genomic full-length RNAs and TMV dRNAs. Derivatives of TMVΔCla dRNA were created with 3′ NTRs from other tobamoviruses, viz., ORSV, TMGMV U2, and TMGMV U5 (Fig. 3A). Substitution of the ORSV 3′ NTR into full-length TMV RNA caused an ~70% reduction in replication (lane 2, Fig. 3B). However, the accumulation of the hybrid ΔCla-like dRNA with the ORSV 3′ NTR could not be detected (lane 2, Fig. 3C). The U2 3′ NTR essentially failed to function for replication of either full-length TMV RNA (lane 3, Fig. 3B) or dRNA (lane 5, Fig. 3C). Substitution of the U5 3′ NTR into full-length TMV RNA resulted in an ~50% reduction in replication of genomic RNA (lane 4, Fig. 3B) and ~60% reduction was observed with *in trans* replication of the dRNA. TMVΔCla/U5-NTR (lane 7, Fig. 3C).

Although hybrid dRNAs with heterologous 3′ NTRs resulted in reduced or no accumulation, increasing the

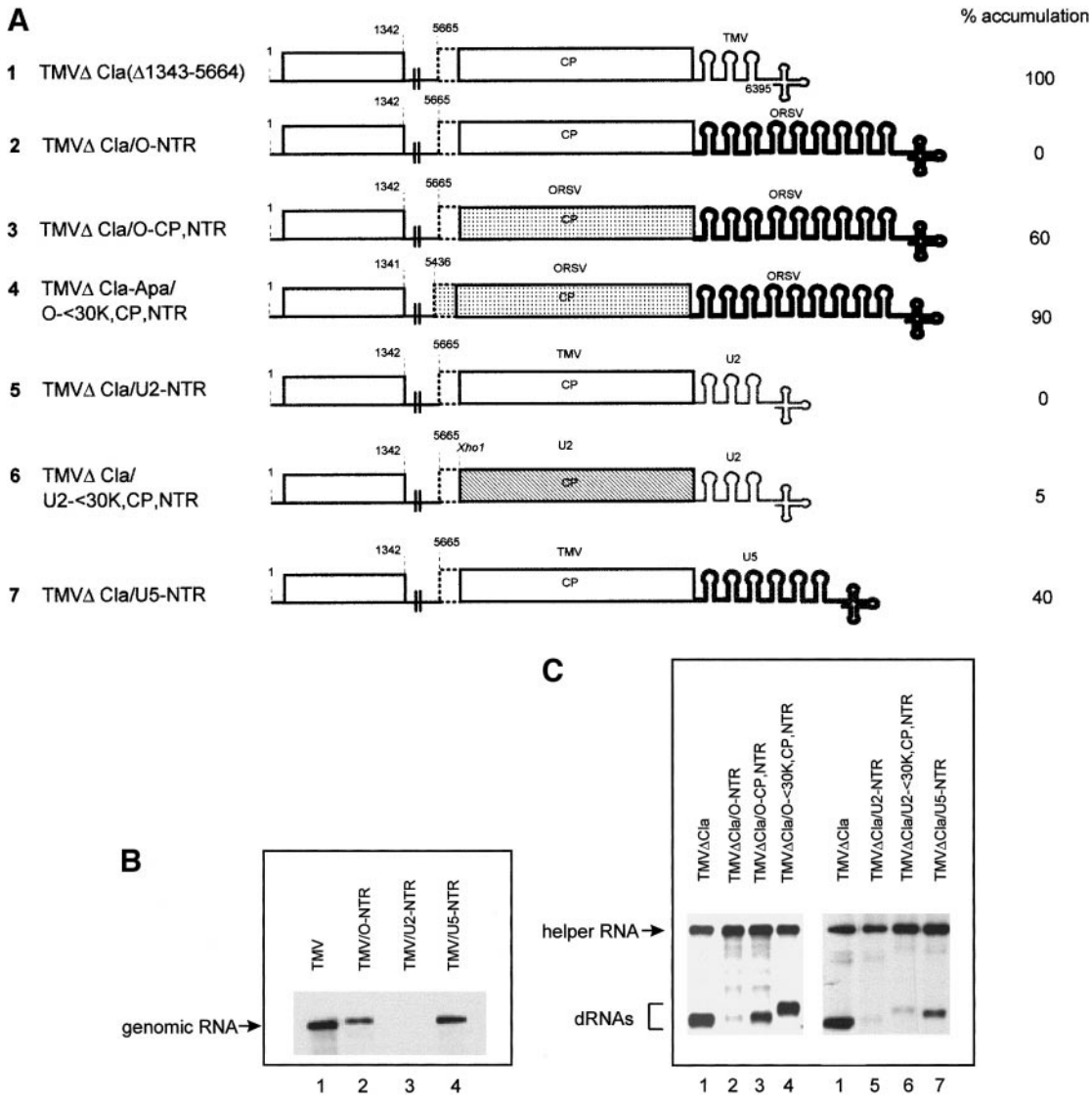

FIG. 3. (A) Schematic diagram of the genomic organization of dRNAs of TMV containing heterologous 3′ sequences. The sequences between nts 1343 and 5664 were deleted. The portion of the genome 5′ of the deletion is not drawn to same scale as the 3′ portion. CP, coat protein ORF. The deletion is shown as +. (B) Accumulation of genomic RNAs of TMV and TMV chimeras with heterologous 3′ NTRs. (C) Accumulation of dRNAs of TMV and TMV chimeras having heterologous 3′ sequences in tobacco suspension protoplasts. Northern hybridization blots of total RNA extracted from protoplasts 20 h postinoculation were probed as described in Fig. 1. *In vitro* transcripts of pT7S3-28 were used as helper RNA for *in trans* replication of dRNAs. The results presented in B are for replication of full-length genomes with heterologous 3′ NTRs. The lane numbers in C correspond to the numbers of the constructs in A. The percentages of accumulation of the different dRNA mutants compared to wild type (100%) are indicated to the right in A.

amounts of contiguous heterologous sequences in the hybrid dRNAs resulted in higher levels of replication. Substitution of the ORSV 3′ NTR plus the coat protein ORF of ORSV into TMVΔCla revived its replication, resulting in a dRNA that was more fit and replicated, ~60%, as well as wild-type TMVΔCla (lane 3, Fig. 3C). Substitution of even more contiguous ORSV sequences into the TMVΔCla-like dRNA (TMV/O-<30K,CP,NTR), such that all 3′ sequences were from ORSV, resulted in a dRNA that was efficiently replicated, ~90% (lane 4, Fig. 3C), as well as the wild-type dRNA. Similarly, more contiguous U2 sequences revived replication of the nonreplicating TMVΔCla/U2-NTR hybrid to detectable levels (lane 6, Fig. 3C).

Examination of the role of the terminal 28 nucleotides on replication of dRNAs

The above results suggest that the 3′ region of the TMV 3′ NTR is primarily involved with the ability of dRNAs to be replicated and that homologous sequences apparently interact better with the replicase complex. A comparison of the sequences of four different tobamoviruses, TMV, ORSV, U2, and U5, showed that the 3′ ter-

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          30          20          10          1
           .           .           .          .
TMV   UUC GAA UCC -CC CCG UUA CCC CCG GUA GGG GCC CA
ORSV  ... ... .U. -.. ..- ... ..U .G. ... .A. ... ..
U2    ... ... ... U.. ..- .A. ..G .C. ... .C. ... ..
U5    ... ... ... U.. ..- .A. ..G .G. ... .C. ... ..
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FIG. 4. 3′ terminal nucleotides of four tobamoviruses. Nucleotide sequence comparison of the 3′ 37–38 nucleotides of TMV, ORSV, TMGMV U2, and TMGMV U5 showing regions of similarity and nucleotide differences. −, Deletion of a nucleotide.

minal region was one of the more conserved regions with only a few nucleotide differences. A conserved hexamer with a restriction endonuclease cleavage site (*Bst*BI) in the cDNA, corresponding to nucleotides 29 to 34 (in TMV, U2, and U5) and 28 to 33 (in ORSV) (numbering from the 3′ terminus) (Fig. 4), facilitated the production of hybrid dRNAs with heterologous 3′ NTR sequences on each side of this cleavage site. To examine the role of the 3′ terminal 28 nucleotides on dRNA replication, we constructed a series of TMVΔCla derivatives in which the 3′ 28 nucleotides of TMV RNA were replaced with the terminal 27 nucleotides of ORSV RNA or terminal 28 nucleotides of TMGMV U2 RNA or TMGMV U5 RNA (Fig. 5A) and assayed for the ability to be replicated by the helper, S3-28. The TMV dRNA with the terminal 28 nucleotides of U5 RNA, which differed from TMV RNA at four positions in this sequence, and by an insertion and a deletion, replicated to levels similar to that of the parent dRNA, TMVΔCla (lane 4, Fig. 5B). In contrast, the dRNA with terminal nucleotide sequences of TMGMV U2 RNA failed to be replicated (lane 3, Fig. 5B), even though it differed from U5 RNA by only a single nucleotide (Fig. 4). The dRNA with the ORSV terminal sequence (Fig. 5A) was also not replicated (lane 2, Fig. 5B).

The dRNAs with the terminal nucleotides of ORSV RNA or TMGMV U2 RNA (lanes 2 and 3, Fig. 5B) had the same replication phenotypes as the dRNAs with the total NTRs from ORSV or TMGMV U2 (lanes 2 and 5, Fig. 3C). To examine whether substitution of a functional 3′ terminal sequence could rescue nonreplicating dRNAs, we exchanged the 3′ 27 nucleotides at the terminus of TMVΔCla/O-NTR with the 3′ terminal 28 nucleotides of TMV RNA. The two sequences differ by four nucleotides and one nucleotide deletion in the ORSV sequence (Fig. 4). TMVΔCla/O-NTR-T-28nts had the TMV sequence through the coat protein ORF followed by the ORSV 3′ NTR, including the nine upstream pseudoknots and most of the TLS (Fig. 5A), except that the terminal 27 nucleotides of the aminoacyl acceptor arm were replaced with the corresponding 28 nucleotides of TMV RNA. This chimera, which retained 385 nucleotides of the heterologous ORSV 3′ NTR, was replicated ~40% (lane 6, Fig. 5B) as much as wild-type TMVΔCla.

Although TMGMV U2 and TMGMV U5 3′ NTRs differ in length by almost 147 nucleotides, which includes three additional pseudoknots, the terminal 28 nucleotides differ by only a single nucleotide (G13 in U5 and C13 in U2) (Fig. 4). TMVΔCla/U2-NTR was not replicated (lane 5, Fig. 3C), whereas TMVΔCla/U5-NTR was (lane 7, Fig. 3C). A hybrid produced by substituting the 3′ 28 nucleotides from U5 3′ NTR into TMVΔHinc-Cla/U2-NTR resulted in the construction of TMVΔHinc-Cla/U2-NTR-U5-28nts (Fig. 5A), which was replicated by the helper virus more efficiently (lane 8, Fig. 5B). Changing the single nucleotide, which was different in the terminal 28 nucleotides between TMGMV U2 RNA and TMGMV U5 RNA, in TMVΔHinc-Cla/U2-NTR, resulted in a dRNA which was more fit to be replicated.

Examination of the pseudoknot in the aminoacyl acceptor arm of the TLS

The sequence differences between the 3′ terminal regions of the dRNAs that were replicated and those that failed to be replicated suggested that the structure of the terminal 28 nucleotides might have a role in initiation of minus-strand RNA synthesis. Approximately 40 3′ nucleotides make up the aminoacyl acceptor arm of the TLS, with a pseudoknot within the terminal 23 nucleotides (Felden *et al.,* 1996). A comparison of this region of the TLSs of the four tobamoviruses showed only 5 or 6 nucleotide differences between the viruses (Fig. 4). The nucleotide sequence of the replicated U5 hybrid RNA compared to the nonreplicated U2 hybrid RNA differed only at nucleotide 13 (G in U5; C in U2). TMV RNA and ORSV RNA had nucleotide differences at positions 7, 13, 15, and 27 (from the 3′ terminus), in addition to a deletion in the ORSV sequence at position 21. Proposed secondary structures for the 3′ 37–38 nucleotides of the RNAs of TMV and TMV chimeras with ORSV, TMGMV U5, or TMGMV U2 NTRs are depicted in Fig. 6A.

One obvious difference was that the terminal sequences of RNAs that were replicated (which had TMV or U5 termini) had three G–C bonds in the pseudoknot in the TLS (between G11 and C23, G12 and C22, G13 and C21 in TMV, and G11 and C23, G12 and C22, G13 and C21 in U5) (Figs. 4 and 6A), whereas the pseudoknots in U2 and ORSV TLS had only two G–C bonds (between G11 and C23, G12 and C22 in U2, and G11 and C22, G12 and C21 in ORSV). To examine the importance of this observation, we created two TMV mutants with only two G–C bonds and mutants of TMV/U2-NTR and TMV/O-NTR with three G–C bonds in the aminoacyl acceptor arm pseudoknot. Figure 6B shows that when the number of bonds in TMV was reduced from three to two, mutants designated TMVCCc (G21→c) and TMVgCg (C23→g) (Fig. 6A), replication of full-length RNAs decreased by ~50–80% (lanes 2 and 3, Fig. 6B), whereas the same mutations in the corresponding dRNAs almost totally abolished replication *in trans* (lanes 2 and 3, Fig. 6C).

Introduction of a mutation in the aminoacyl acceptor arm of ORSV TLS so that the number of GC bonds was

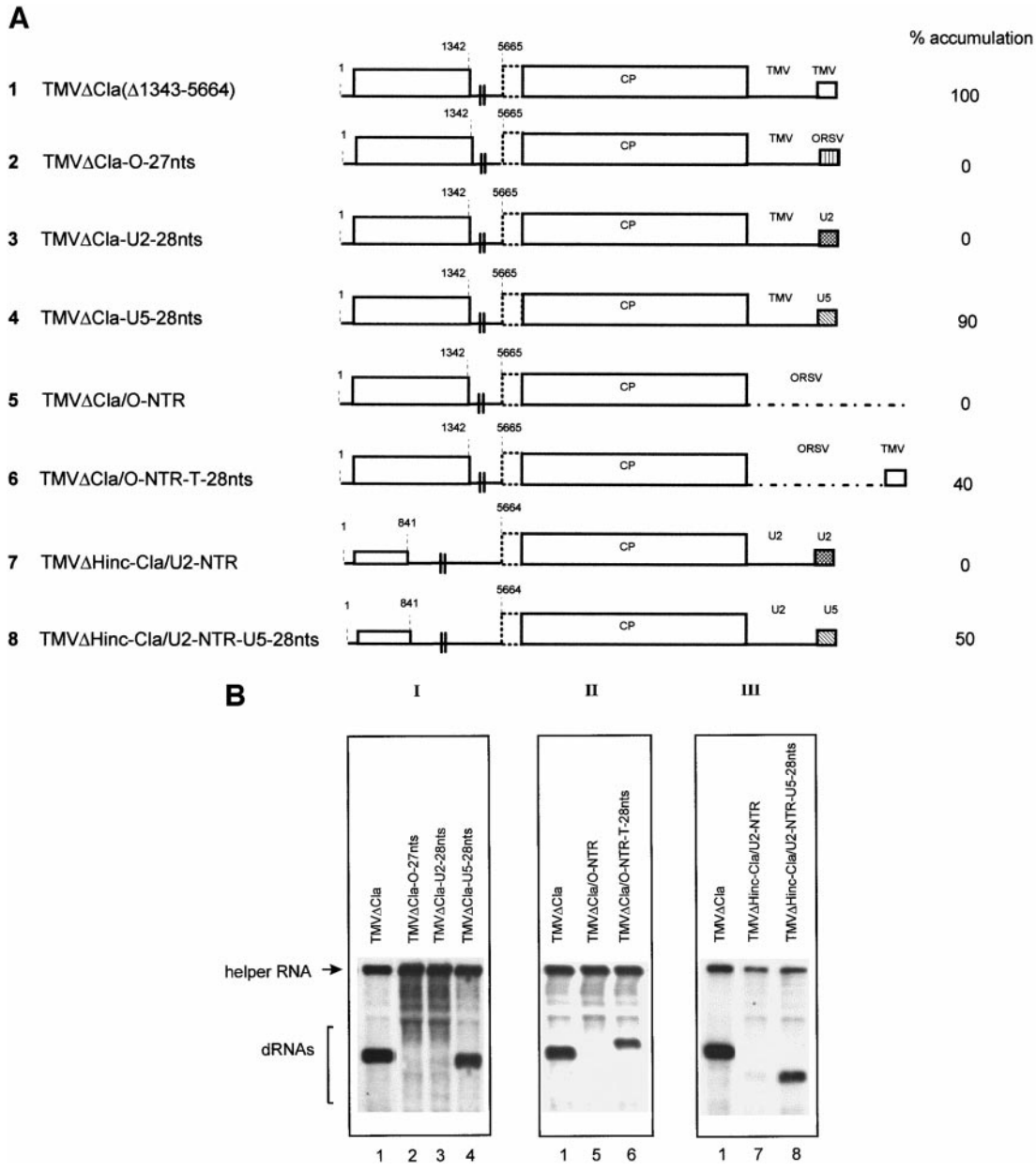

FIG. 5. (A) Schematic diagram of the genomic organization of dRNAs of TMV with the last 28 nucleotides substituted. The sequences between nts 1343 and 5664 were deleted. The portion of the genome 5′ of the deletion is not drawn to the same scale as the 3′ portion. CP, coat protein ORF. The deletion is shown as ∓. (B) Accumulation of dRNAs of TMV, and TMV chimeras with heterologous 3′ terminal sequences, in tobacco suspension protoplasts. Frames I, II, and III show the different sets of chimeras: I depicts replication of dRNA TMVΔCla with the last 28 nts replaced with sequences from ORSV, U2, U5; II depicts replication of dRNA TMVΔCla/O-NTR and a chimera with the last 27 nts replaced with sequences from TMV; and III depicts replication of dRNA TMVΔHinc-Cla/U2-NTR and a chimera with the last 28 nts replaced with sequences from U5 RNA. Northern hybridization blots of total RNA extracted from protoplasts 20 h postinoculation were probed as described in Fig. 1. *In vitro* transcripts of pT7S3-28 were used as helper RNA for *in trans* replication of dRNAs. The times of exposure of the hybridization blot differ between the sets. The lane numbers in B correspond to the numbers of the constructs in A. The percentages of accumulation of the different mutants compared to wild type (100%) are indicated to the right in A.

increased from two to three (when U20→c) resulted in an ~50% increase in replication of TMV/O-NTR-CCc *in cis* (lane 5, Fig. 6B) over the parent chimera TMV/O-NTR (lane 4, Fig. 6B). There was a small increase in the level of accumulation of the dRNA TMVΔCla/O-NTR-CCc (lane 5, Fig. 6C) over TMVΔCla/O-NTR. Similarly, mutation of the sequence in the aminoacyl acceptor arm of U2 RNA bringing the number of bonds in the pseudoknots from two to three (when C21→g), but designed not to recreate U5 RNA, resulted in an increase in the levels of replication. Full-length TMV/U2-NTR RNA did not replicate (lane 6, Fig. 6B), although introduction of the mutation in the TLS pseudoknot (Fig. 6A) caused the accumulation of TMV/U2-NTR-CCg genomic RNA to increase substan-

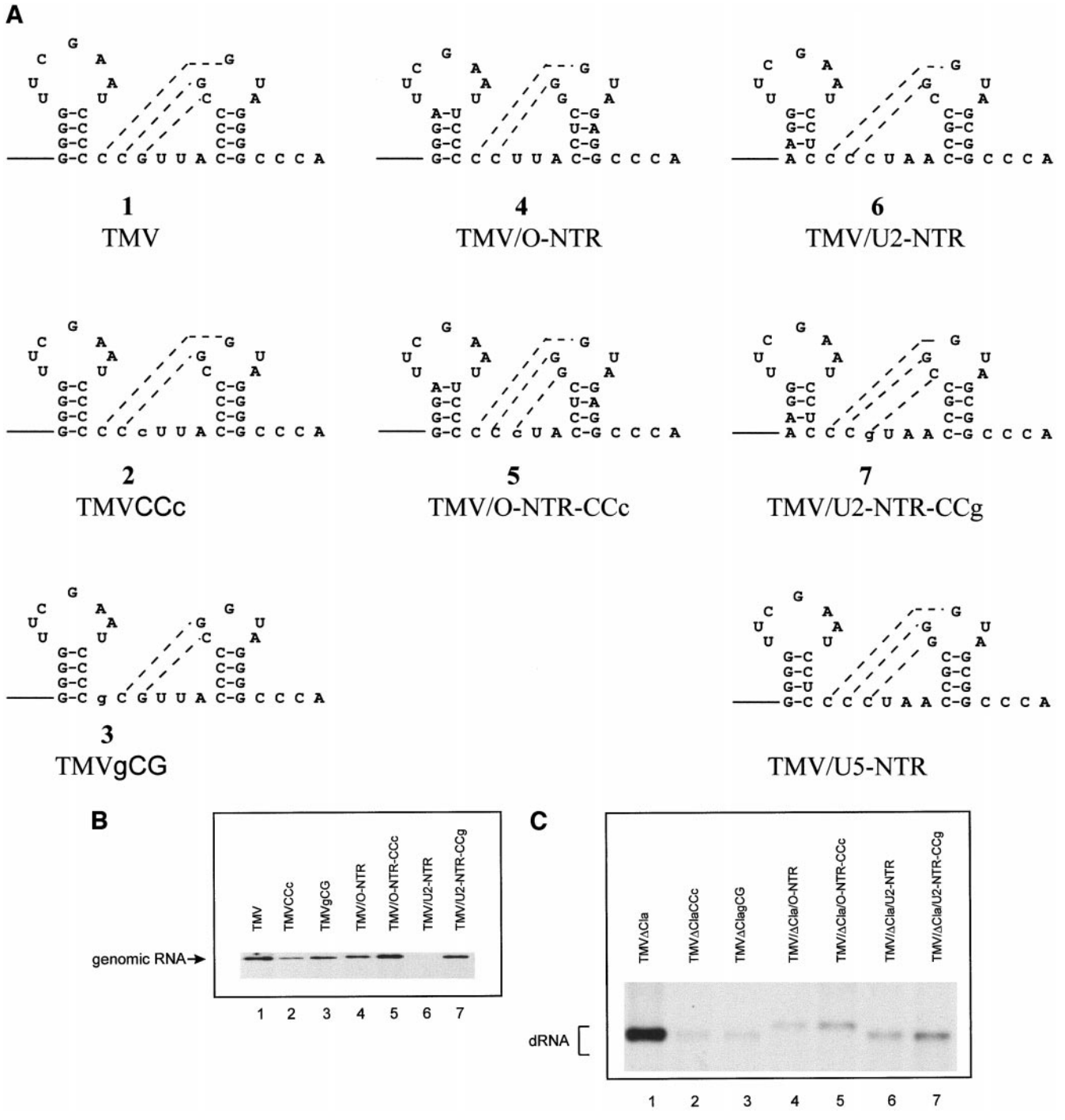

FIG. 6. (A) Proposed secondary structures of the last 37–38 nucleotides of RNAs of TMV and TMV chimeras with heterologous 3′ NTRs. Point mutations are depicted in lowercase letters. G–C bonds are shown as —. (B) Accumulation of genomic RNAs of TMV and TMV chimeras with mutations in the pseudoknot of the aminoacyl acceptor arm in the TLS and (C) accumulation of dRNAs of TMV and TMV chimeras with mutations in the pseudoknot of the aminoacyl acceptor arm in the TLS. Northern hybridization blots of total RNA extracted from protoplasts 20 h postinoculation were probed as described in Fig. 1. *In vitro* transcripts of pT7S3-28 were used as helper RNA for *in trans* replication of dRNAs. The lane numbers in B correspond to the numbers of the constructs in A. The lanes in C correspond to ΔCla derivatives of the constructs represented in B.

tially to ~40% of TMV RNA (lane 7, Fig. 6B). The dRNA, TMVΔCla/U2-NTR-CCg, with the same mutation, also was replicated more efficiently (lane 7, Fig. 6C), although the level was much lower than that of TMVΔCla (lane 1, Fig. 6C).

DISCUSSION

In earlier studies we observed that defective full-length TMV RNAs were not replicated *in trans* (Lewandowski and Dawson, 1998), suggesting that genomic

RNA of TMV is probably replicated *in cis* by a coupled translation–replication mechanism in which nascent replicase protein initiates synthesis of negative-stranded RNAs, as has been proposed for other positive-stranded RNA viruses (Kusov *et al.,* 1996; Lai, 1998; Dreher, 1999). However, TMV dRNAs with certain internal sequences removed were replicated efficiently *in trans,* to levels as high as that of the helper virus RNA. The replication of the dRNAs did not affect the level of replication of the helper RNA, suggesting that the dRNAs probably used excess replication complexes, perhaps from a different pool, not needed by the helper virus RNA. Thus, there appear to be different rules for engagement between replicase complexes and different RNAs (Lewandowski and Dawson, 1998).

The 3′ NTR of TMV and the similar ToMV consists of a TLS and three upstream pseudoknots (van Belkum *et al.,* 1985; Pleij *et al.,* 1987). It previously was shown that the tRNA-like region and the 3′-most pseudoknot (pk3) constitute the core replication signal for genomic ToMV RNA replication (Takamatsu *et al.,* 1990). The 5′ pseudoknot (pk1) and middle pseudoknot (pk2) are not absolutely required for replication of genomic ToMV RNA, but greatly affect efficiency of replication. Deletion into pk1 in ToMV RNA decreased replication to ~70% of the wild-type level while deletion into pk2 resulted in replication only 10–30% that of wild-type levels (Takamatsu *et al.,* 1990). When precise removal of the TMV pseudoknots was made, it was observed that deletion of pk1 and/or pk2 (Fig. 2B) resulted in reduced replication, and deletion of pk3 almost totally prevented replication, essentially the same as that of ToMV. However, these deletions had different effects on the ability of dRNAs to be replicated *in trans.* Although the absence of pk1 and/or pk2 affected the level of replication of genomic RNAs, it had little or no effect on the replication of dRNAs (Fig. 1B). Near-optimal levels of replication of the dRNAs were observed with a small section of the 3′ NTR, consisting of only pk3 and the tRNA-like element (lane 8, Fig. 1B).

Different tobamoviruses have 3′ NTRs containing a 5′ pseudoknot region and a 3′ tRNA-like region. All of the TLSs charge histidine *in vitro* except sunnhemp mosaic virus (SHMV) which charges valine (Garcia-Arenal, 1988). Heterologous 3′ NTRs generally are functional for replication of genomic RNAs. Chimeric ToMV genomic RNAs containing substitutions of 3′ NTRs from TMV, cucumber green mottle mosaic virus, or SHMV resulted in continued replication, although the divergent NTRs resulted in substantially reduced replication (Ishikawa *et al.,* 1988). Likewise, chimeric TMV RNAs with ORSV 3′ termini replicated efficiently (Hilf and Dawson, 1993) as did TMV-based expression vectors with ToMV, U2, or U5 3′ NTRs (Shivprasad *et al.,* 1999). In fact, genomic RNAs with duplicate 3′ NTRs in tandem (ORSV/TMV: Hilf and Dawson, 1993; U5/TMV: Shivprasad *et al.,* 1999) also replicated efficiently. Genomic RNAs with 3′ NTRs from even the most divergent tobamoviruses based on sequence similarity replicated to some extent, and hybrid genomic RNAs with many of the heterologous 3′ NTRs replicated almost as well as viruses with their homologous 3′ NTRs. In contrast, heterologous 3′ sequences had a greater effect on dRNA fitness. For example, the ORSV 3′ NTR which was lethal to the dRNA (lane 2, Fig. 3C) did not have such a dramatic effect on genomic RNA synthesis (lane 2, Fig. 3B), and the U5 3′ NTR worked better for replication of genomic RNAs (lane 4, Fig. 3B) than for replication of the hybrid dRNA (lane 7, Fig. 3C). These results demonstrate that even though the replication signals of the dRNAs were smaller than that of the genomic RNAs, the sequence specificity was greater.

An extraordinary feature of the *in trans* interaction of the replication complexes with the heterologous 3′ *cis*-acting elements was that additional heterologous sequences were compensatory. Additional contiguous heterologous sequences, including the coat protein ORF and part of the movement protein ORF (Fig. 3C), resulted in much greater fitness of the dRNA. The larger 3′ sequences from ORSV and U2 revived replication. These results suggest that even though the minimal replication signals are contained within the 3′ 135 nucleotides, these elements normally are affected by the context of surrounding sequences and structures.

The remarkably similar forms of TMGMV, U2 and U5, differ primarily in their 3′ NTRs, with the pseudoknot regions being the most dissimilar. Since chimeric dRNAs containing heterologous sequences at the 3′ ends differed dramatically in their abilities to be replicated, the specificities for being recognized by replicase complexes mapped to the 3′ 28 nucleotides instead of the divergent upstream pseudoknot region. For example, the chimeric dRNA with the ORSV 3′ NTR had six more pseudoknots than TMV RNA in the pseudoknot region and failed to be replicated. However, replacement of the 3′ 28 nucleotides (not the 5′ sequences including the divergent pseudoknot region) with those of TMV RNA resulted in a revival of dRNA replication (lane 6, Fig. 5B). Even though this chimeric dRNA, TMVΔCla/O-NTR-T-28nts, had a heterologous pseudoknot region and tRNA-like region except for the acceptor arm (the 3′ 28 nts), it was replicated efficiently. Despite the fact that the rest of the TLS and at least part of the upstream pseudoknot region were required for the dRNAs to be replicated, the specificity appeared to be within the terminal nucleotides, specifically within the aminoacyl acceptor arm of the TLS.

The 3′ 28 nucleotides in tobamoviruses constitute a stem loop and four free 3′ nucleotides, similar to the minimal promoter element characterized for replication of the satellite RNA of turnip crinkle virus (Song and Simon, 1995), except that the tobamovirus loop binds to an upstream sequence forming a pseudoknot. Disruption of this pseudoknot in turnip yellow mosaic virus (TYMV;

TABLE 1

Description of Constructs Used in the Study

TMV	[TMV1–6395]-G ↓ GTACC
TMVΔpk1	[TMV 1–6215]-[TMV 6238–6395]-G ↓ GTACC
TMVΔpk2	[TMV 1–6238]-[TMV 6260–6395]-G ↓ GTACC
TMVΔpk3	[TMV 1–6259]-[TMV 6290–6395]-G ↓ GTACC
TMVΔpk1 + pk2	[TMV 1–6215]-[TMV 6260–6395]-G ↓ GTACC
TMVΔCla	[TMV 1–1342]-TMV 5665–6395]-G ↓ GTACC
TMVΔClaΔCP	[TMV 1–1342]-[TMV 5665–5711]-[TMV 6193–6395]-G ↓ GTACC
TMVΔClaΔ1342–6249	[TMV 1–1341]-[TMV 6250–6395]-G ↓ GTACC
TMVΔClaΔ1342–6365	[TMV 1–1341]-[TMV 6366–6395]-G ↓ GTACC
TMVΔClaΔpk1	[TMV 1–1343]-[TMV 5665–6215]-[6238–6395]-G ↓ GTACC
TMVΔClaΔpk2	[TMV 1–1343]-[TMV 5665–6238]-[TMV 6260–6395]-G ↓ GTACC
TMVΔClaΔpk3	[TMV 1–1343]-[TMV 5665–6259]-[TMV 6290–6395]-G ↓ GTACC
TMVΔClaΔpk1 + pk2	[TMV 1–1343]-[TMV 5665–6215]-[TMV 6260–6395]-G ↓ GTACC
TMVΔCla/O-NTR	[TMV 1–1343]-[TMV 5665–6192]-[ORSV 6218–6609]-G ↓ GTACC
TMVΔCla/O-CP,NTR	[TMV 1–1343]-[TMV 5665–5711]-[ORSV 5721–6609]-G ↓ GTACC
TMVΔCla/O-<30K,CP,NTR	[TMV 1–1341]-[ORSV 5436–6609]-G ↓ GTACC
TMVΔCla/U2-NTR	[TMV 1–1343]-[TMV 5665–6192]-CTCGAG-[TMGMV U2 6146–6355]-G ↓ GTACC
TMVΔCla/U2-<30K,CP,UTR	[TMV 1–1343]-[TMV 5665–5714]-CTCGAG-[TMGMV U2 5429–6355]-GATATC-G ↓ GTACC
TMVΔCla/U5-NTR	[TMV 1–1343]-[TMV 5665–6192]-CTCGAG-[TMGMV U5 6146–6502]-G ↓ GTACC
TMVΔCla-O-27nts	[TMV 1–1343]-[TMV 5665–6363]-[ORSV 6578–6609]-G ↓ GTACC
TMVΔCla-U2-28nts	[TMV 1–1343]-[TMV 5665–6363]-[TMGMV U2 6324–6355]-G ↓ GTACC
TMVΔCla-U5-28nts	[TMV 1–1343]-[TMV 5665–6363]-[TMGMV U5 6471–6502]-G ↓ GTACC]
TMVΔCla-O-NTR-T-28nts	[TMV 1–1343]-[TMV 5665–6192]-[ORSV 6218–6577]-[TMV 6364–6395]-G ↓ GTACC
TMVΔHinc-Cla/U2-NTR	[TMV 1–841]-GC-[TMV 5664–6192]-[TMGMV U2-6146–6355]-G ↓ GTACC
TMVΔHinc-Cla/U2-NTR-U5-28nts	[TMV 1–841]-GM-]TMV 5664–6192]-[TMGMV U2 6146–6323]-[TMGMV U5 6471–6502]-G ↓ GTACC
TMVCCc	[TMV 1–6372]-CCc-[TMV 6376–6395]-G ↓ GTACC
TMVgCG	[TMV 1–6372]-gCG-[TMV 6376–6395]-G ↓ GTACC
TMV/O-NTR-CCc	[TMV 1–6192]-[ORSV 6218–6587]-CCc-[ORSV 6591–6609]-G ↓ GTACC
TMV/U2-NTR-CCg	[TMV 1–6192]-[TMGMV U2 6146–6332]-CCg-[TMGMV U2 6336–6355]-G ↓ GTACC
TMVΔCla-CCc	[TMV 1–1343]-[TMV 5665–6372]-CCc-[TMV 6376–6395]-G ↓ GTACC
TMVΔCla-gCG	[TMV 1–1343]-[TMV 5665–6372]-gCG-[TMV 6376–6395]-G ↓ GTACC
TMVΔCal/O-NTR-CCc	[TMV 1–1343]-[TMV 5665–6192]-[ORSV 6218–6587]-Ccc-[ORSV 6591–6609]-G ↓ GTACC
TMVΔCla/U2-NTR-CCg	[TMV 1–1343]-[TMV 5665–6192]-[TMGMV U2 6146–6332]-CCg-[TMGMV U2 6336–6355]-G ↓ GTACC

Note. Virus sources and nucleotides are indicated between brackets. Additional nucleotides are indicated. ↓ Indicates the end of *in vitro* transcripts after linearization of the plasmid DNA with *Kpn*I.

Deiman *et al.,* 1997), brome mosaic virus (Dreher and Hall, 1988; Chapman and Kao, 1999) and alfalfa mosaic virus (Olsthoorn *et al.,* 1999) results in a reduction of minus-strand promoter activity. Gargouri-Bouzid *et al.* (1991) showed that for TYMV, the entire TLS was not essential for negative-strand synthesis but a terminal 38 nucleotide fragment was sufficient for initiation of replication. Further mapping of the 3′ promoter in TYMV RNA revealed that fragments as short as 28 nucleotides could act as templates for negative-stranded RNA synthesis, and minimal *in vitro* promotion required only the terminal three nucleotides (Singh and Dreher, 1998), although, *in vivo,* a larger replication signal was required. It has been suggested that the TLS in TYMV RNA is required for presenting the 3′ CCA in a conformation that is easily accessible to the replicase (Dreher, 1999).

Examination of the pseudoknot in the acceptor arm of the TLS of these tobamoviruses demonstrated that RNAs with sequences that allowed three G–C bonds between the loop region and sequences outside the loop were replicated by the TMV replicase, both *in cis* and *in trans,* whereas those with only two G–C bonds were replicated poorly or failed to be replicated (Figs. 6A and 6B). In fact, the difference of a single nucleotide between U2 and U5, involved in binding in this region, was sufficient to turn replication off or on. However, all of these 3′ terminal sequences are efficiently recognized by their homologous replication complexes. These data demonstrate differences between the different tobamovirus replicase complexes.

It is likely that the differences in requirements of the replicase complexes from different tobamoviruses could also be extended to *in trans* replication of dRNAs. Although we observed that the TMV replicase complex did not function effectively *in trans* with the termini of ORSV or U2 RNAs, the ORSV and U2 enzyme complexes would be expected to be compatible with the homologous termini. In fact, we have found that ORSV does efficiently replicate ORSV-derived dRNAs (unpublished data).

In summary, the 3′ NTR requirements for optimal replication of TMV RNAs *in cis* and *in trans* were different. Efficient replication of dRNAs required a smaller section

of the 3′ terminal region than did replication of the helper virus RNA. On the other hand, replication of dRNAs required more precise homology between replication complexes and the acceptor arm of the TLS. If the 3′ replication signals were defined by *in cis* versus *in trans* requirements for replication, it would suggest that the replicase complex–RNA interactions were different. Perhaps presentation of nascent replicase protein to the 3′ end requires different replication signals than are needed by the replication complexes from the preassembled pool used by dRNAs.

MATERIALS AND METHODS

Plasmid DNAs and *in vitro* transcription

Details of all constructs are outlined in Table 1. All TMV clones were derived from an infectious cDNA clone of TMV (Dawson *et al.,* 1986) with the T7 promoter fused to the 5′ end of the TMV sequence as described previously (Lewandowski and Dawson, 1998). The nucleotide numbering is according to Goelet *et al.* (1982). The procedures used in cloning were essentially those described by Sambrook *et al.* (1989). When required, after restriction endonuclease digestion, the DNA was blunt-ended using mung bean nuclease and the blunt ends were religated. All of the dRNAs, except TMVΔHinc-ClaI/U2-NTR and TMVΔHinc-ClaI/U2-NTR-U5-28nts, were ΔClaI derivatives of full-length clones that were obtained by digesting plasmid DNA with *Cla*I and religating the larger fragment. This resulted in a deletion of nucleotides 1343–5664 of the TMV genome. pT7S3-28 which is derived from pTMVS3-28 (Dawson *et al.,* 1998) was used as a helper for studying *in trans* replication of dRNAs (Lewandowski and Dawson, 1998).

Pseudoknot deletions in the 3′ NTR of TMV were made according to the boundaries described by Gultyaev *et al.* (1994). The three pseudoknots are described from 5′ to 3′ as pk1, pk2, and pk3. Overlap-extension-polymerase chain reaction (PCR) using appropriate primers and Vent DNA polymerase (New England Biolabs, Beverly, MA) was used in the construction of the full-length mutants. ΔClaI derivatives were constructed by cloning the *Nsi*I–*Kpn*I fragment, containing the deletion, into similarly digested TMVΔClaI.

Mutants with heterologous 3′ NTRs, and those with a single point mutation in the aminoacyl acceptor arm of the tRNA-like structure of the 3′ NTRs, were constructed using PCR with appropriate primers and Vent DNA polymerase (New England Biolabs). The templates used in the PCR reaction were cDNA clones of TMV chimeras with *Odontoglossum* ringspot virus (Hilf and Dawson, 1993), tobacco mild green mosaic virus strain U2, and tobacco mild green mosaic virus strain U5 (Shivprasad *et al.,* 1999). TMVΔHinc-ClaI/U2-NTR was constructed by digesting TMV/U2-NTR with *Hin*cII and *Cla*I, filling the single-stranded overhang with the Klenow fragment of *Escherichia coli* DNA polymerase I (New England Biolabs) and religating the plasmid. This resulted in deletion of nucleotides 842–5663 of the TMV genome. The last 28 nucleotides of the U5-NTR (from TMV/U5-NTR) were introduced as a *Bst*BI–*Kas*I fragment into similarly digested TMVΔHinc-ClaI/U2-NTR to construct TMVΔHinc-ClaI/U2-NTR-U5-28nts.

The sequences of all of the constructs were confirmed by DNA sequencing at the ICBR sequencing core facility at the University of Florida.

Protoplast transfection and RNA analysis

Protoplasts from a *N. tabacum* cv. Xanthi suspension cell line were transfected with *in vitro* generated transcripts by the procedure described previously (Lewandowski and Dawson, 1998). *In vitro* transcripts of pT7S3-28 (Lewandowski and Dawson, 1998) were used as helper RNA in experiments examining *in trans* replication. Protoplasts were coinoculated with helper and dRNA in a 1:1 ratio of transcription reaction product. The protoplasts were incubated for 20 h and total RNA was extracted and analyzed by Northern blot hybridization by the procedure described previously (Lewandowski and Dawson, 1998). A digoxigenin-labeled positive-strand-specific riboprobe [5′(+)-specific], which reacts with the 5′ 256 nucleotides of the TMV genome, was used to detect replication according to the manufacturer's specifications (Roche Molecular Biochemicals, Indianapolis, IN). For quantitative estimation of RNA, the X ray films were scanned, and densitometric analysis was carried out using the OS-Scan image analysis software (Oberlin Scientific). The data presented are from five replicate experiments.

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